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Breast cancer results from inappropriate cell proliferation due to mutations that disrupt normal cell cycle control. Our studies are focused on understanding the role of the p21Cip1 protein in cancer. p21 is a member of a growing family of proteins which bind and inhibit cyclin dependent kinases (Cdks). Cdks are required for cell cycle progression. p21 is regulated by the tumor suppressor protein p53 and is thought to contribute to p53's tumor suppressor function through its interaction with Cdks. p53 is commonly mutated in breast cancer. We have examined the expression of p21 during development and in adult tissues and have found that p21 expression is highly selective and correlates with terminal differentiation. We have also analyzed the phenotype of mice lacking p21. We have found that p21 is responsible for only a subset of p53's function. Specifically, p21 is required for G1 checkpoint function but is not required for the G2 checkpoint or apoptosis induction. Moreover, animals lacking p21 do not readily develop tumors, implying that p21 is not a tumor suppressor. Future studies will involve further analysis of checkpoint functions and will examine genetic interactions between oncogenes and loss of p21 in vivo.

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J. Wade Harper
Principal Investigator's Signature

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Introduction

Proliferation of eukaryotic cells is regulated primarily at two points in the cell cycle, in G1 prior to entry into S-phase and in G2 prior to entry into mitosis. The decision of whether to commit to a round of cell division or exit the cell cycle is made at a point in G1, referred to as the restriction point in mammalian cells[1-4] or START in yeast[5]. In fibroblasts, passage through the restriction point depends critically on the signals that are received through mitogen activated pathways, but once this point is passed, cells are committed to S-phase and the remainder of the cycle in a mitogen independent manner[4]. In somatic tissues, passage through the restriction point is thought to be the primary event controlling cell proliferation. Therefore, elucidating how positively and negatively acting genes function to regulate the G1/S transition and how mutations in these genes disrupt normal cell cycle control has been a primary focus of cancer research. Central to this focus has been the investigation of the role of cyclin-dependent kinases (Cdk) in the control of cell proliferation.

Cyclins, Cdks, and positive growth control. Cdks are protein kinases that require association with cyclins and phosphorylation for activity[5-7]. Currently, there are seven *bone fide* Cdks and several others related by sequence[8]. Biochemical and genetic data from several systems have demonstrated that cyclins promote cell cycle transitions via their ability to associate with and activate their cognate Cdks[5-12]. D-type and E-type cyclins function in the G1 phase of the cell cycle[6, 10, 13-16], and overexpression of cyclin D1 or cyclin E shortens G1 and accelerates entry into S-phase[1, 3, 17, 18]. A large body of correlative evidence exists linking deregulated cyclin expression to cancer. Amplification of cyclins, D1, D2 and E have been identified in several tumors[19-23]. Cyclin D1 was identified as the PRAD1 oncogene [24]. Cyclin A was identified as the site of integration of HBV in a hepatocellular carcinoma[25]. Taken together, these observations suggest that inappropriate activation of Cdks is a mechanism that cells frequently use to reach the oncogenic state.

D-type cyclins associate with Cdk4 and Cdk6 kinases and can phosphorylate and inactivate Rb[6, 26-29]. Since D-type cyclins are required for proliferation of tissue culture cells only if they have an intact Rb gene, it is thought that Rb inactivation is their primary role, however this has not been rigorously demonstrated in animals. Cyclin E binds to and activates Cdk2 and considerable evidence has accumulated indicating that cyclin E/Cdk2 is the primary kinase involved in the G1/S transition[14, 15, 30-33]. In addition, a close homolog of Cdk2 - Cdk3 - is also thought to play a unique role in the G1/S transition[31]. Cyclin A binds Cdk2 and Cdc2 and is required for both S-phase and the G2/M transition [34-36], while cyclin B/Cdc2 complexes appear to be specific for control of mitotic entry.

Since the controls utilized during development to regulate cell proliferation are similar to those utilized in maintenance of the non-proliferative state in differentiated tissues, it is likely that these controls are reactivated or overcome in cancer. A primary example of this comes from our observation that cyclin D1 is expressed at extremely high levels in the retina and is required for its development [37], as demonstrated by the fact that mice lacking cyclin D1 have a major defect in retinal cell proliferation during development. Presumably the inability to properly develop the retina in cyclin D1 mutants reflects an inability to overcome Rb. In what is clearly more than a coincidence, the retina is the same tissue in which high frequency tumors arise in Rb mutant humans. It is therefore likely that the Rb protein is important in both development of that tissue and its maintenance in the non-proliferative state. Our understanding of the links between development and cancer is in its infancy and is an area in which there is a great need to increase our knowledge base.

Tumor suppressor proteins and negative growth control. Much of our knowledge about negative regulation of cell cycle entry emanates from studies of two tumor suppressor genes, Rb and p53[29, 38]. Mutations in these are found frequently in diverse types of human cancers[39,40], and reintroduction of wild-type genes into $p53^-$ or Rb^- tumor cells can suppress the neoplastic phenotype suggesting that loss of function of these genes contributes to tumorigenesis[28, 41, 42].

Mutations in p53 are the most common lesions observed in human malignancies, occurring in greater than 50% of all tumors[39] including those of the breast. The percentage is much higher if loss of p53 function via association with viral oncoproteins (E1B of adenovirus and E6 of papilloma virus) or amplification of the p53 binding protein MDM2 are included[43]. p53 deficient mice are prone to the spontaneous development of a variety of tumor types[44]. Cellular responses to DNA damage such as apoptosis and the G1 checkpoint are dependent on p53[45-53]. p53 also controls a spindle checkpoint and prevents genetic alterations such as gene amplification[54, 55].

Much is known about the function of Rb in cell cycle control and the large number of proteins with which it interacts [29]. The current view is that hypo-phosphorylated Rb functions during G1 in part to block the activity of E2F and related transcription factors that are required for the expression of genes involved in S-phase. Hyper-phosphorylation of Rb or association with DNA tumor virus oncoproteins such as E1A results in release of E2F and is correlated with passage into S-phase.

These data are consistent with a model in which increased cyclin/Cdk activity in tumors, whether by increased cyclin expression or decreased negative regulation, can overcome the cell cycle repression function of Rb via direct phosphorylation and inactivation of its growth inhibitory function. Rb therefore acts as a potential energy barrier in the pathway that cyclin/Cdks must overcome to activate cell cycle entry. Removal of the barrier (Rb) may reduce the levels of kinase activity required, but some Cdk kinase activity is still required for the process of DNA replication and can therefore act as a target of further negative regulation. In this model, p53 acts to reduce the frequency of mutations that lead to altered growth control and to kill cells that have undergone extensive damage or are inappropriately growing. To fully understand this aspect of cancer, cell cycle dysfunction, it is imperative that we have a complete understanding of the regulation of cyclin dependent kinases and their regulators in the tissues of interest.

Cyclin-dependent kinase inhibitors: mediators of negative cell cycle control. Cdk inhibitory proteins are a recently discovered group of proteins that associate will and inhibit Cdks. There versatile molecules have potential roles in cell cycle arrest, checkpoint function and development and are likely to cooperate with Rb, p53, and other negative regulators in maintaining the non-proliferative state throughout adult life. At the time of submission of this grant in December 1993, the first mammalian Cdk inhibitors $p21^{CIP1/WAF1}$ (57-600) and $p16^{INK4a}$ (61) had only recently been identified. We identified $p21^{CIP1}$ in a two-hybrid screen designed to identify proteins that associate with Cdk2 (57). Importantly, this protein was simultaneously cloned by several other laboratories. p21 was cloned as a p53 activated gene by the Vogelstein laboratory (59), as a Cdk associated protein by the Beach laboratory (58), and as an S-phase inhibitory cDNA in senescent cells (60). At that time there were many unanswered questions regarding the role p21 in cell cycle control and cancer. What was known was that p21 could bind and inhibit a number of Cdks, that it was transcriptionally regulated by p53 overexpression, and that its chromosomal location did not mark it as an obvious tumor suppressor. In addition, it was not known whether p53 is the only regulator of p21 expression, how p21 might be used during development, or whether loss of p21 expression contributes to cancer.

Since that time, there has been a virtual explosion in the field of CKIs. To date, two additional p21 homologs -$p27^{KIP1}$ and $p57^{KIP2}$ - have been identified (77,78,81,82), and four $p16^{INK4}$ homologs have been identified (61-65). Much is now known about these inhibitors, their association with Cdks and other molecules, their potential roles in development , how they are regulated, and their potential roles as tumor suppressors (79,80). Some of what is known about these inhibitors is summarized in Table 1.

The goals of our work were: 1) to determine the pattern of expression of p21 during embryogenesis and in adult tissues using in situ hybridization, 2) to construct mice lacking p21, 3) to analyze the phenotype of mice lacking p21, 4) to examine the status of p21 in breast tumors and tumor cell lines, and 5) to characterize the regulation of p21 in the cell cycle and to identify interacting proteins. We have made significant progress toward these goals with six published papers relating to p21 function. We have: 1) determined the expression pattern of p21 during

mouse development and in adult tissues and have shown that expression during development is independent of p53 [72], 2) in collaborative studies with Drs. C. Deng and P. Leder, we have analyzed the phenotype of mice lacking p21 and have examined the effects of loss of p21 on checkpoint control [56], 3) in collaborative studies with Dr. J. Hurwitz we have identified PCNA as a p21 associated protein and have found that this association blocks DNA replication in vitro [67] and have studied the interactions of p21 with Cdks in detail [66]. In addition, we have examined the regulation of p21 in response to DNA damage and have found that it accumulates on cyclin E/Cdk2 complexes in responsee to DNA damage [69,70]. The critical aspects of this work are summarized below within the context of the original goals of the grant.

Body

Aim 1: Expression of p21 during development and in adult tissues

p21 is expressed largely in terminally differentiated cell populations. In situ hybridization was used to examine the patterns of expression of p21 during embryonic development and in some adult tissues [72]. The results are summarized in Tables I and II, and have been published [72]. p21 expression early in development is extremely cell type dependent and occurs primarily in cells that are thought to be terminally differentiated, correlating with cell cycle arrest. The most striking expression pattern occurs in ht dermomyotome where p21 induction is paralleled by that of myogenin. Myogenin is a basic HLH transcription factor which functions in the muscle cell differentiation pathway and is expressed in cells that have already committed to the muscle cell differentiation program and are arrested. In addition, the vast majority of cell types indicated in Table I are terminally differentiated and thought to be post mitotic.

p53 can activate p21 expression in response to DNA damage [69,70]. In order to determine whether p53 regulates p21 expression during development, in situ hybridization was performed using embryos from p53 null mice. p53 is expressed widely in the embryo. In p53 null embryos, p21 is expressed in a pattern identical to that found in wild type animals [72]. Similar results were obtained in adult tissues. Thus, our results indicate that p53 does not regulate the normal expression of p21 during development.

p21 is also expressed in a highly selective manner in adult tissues[72]. For example, in the stomach and intestine, p21 expression is limited to the columnar epithelium. These are cells that are known to be differentiated. In addition, p21 is found to be widely expressed in tissues such as lung, liver and muscle.

Our results suggest that p21 may function in cellular differentiation. To examine this further in an experimentally characterized system, we studied the expression of p21 during muscle cell differentiation in vitro [72]. The myogenic program is controlled by the transcription factor MyoD and MyoD or its homolog myf5 are sufficient to convert a number of cell types into muscle. We found that p21 is transcriptionally activated in response to activation of MyoD in fibroblast. In these cells, MyoD is stabily expressed and kept inactive by growth factors present in the serum. When serum is removed, MyoD is activated and p21 mRNA accumulates. In addition, we found that p21 is induced during differentiation of C2 cells. C2 myoblasts contain MyoD in an inactive form and removal of serum leads to the formation of multinucleated post-mitotic myotubes. p21 mRNA is induced in a temporal manner essentially indistinguishable from myogenin in these cells. Interestingly, while MyoD activation can lead to p21 induction in vitro, mice lacking MyoD still express p21, suggesting a possible redundant role of myf5. Similar results were obtained independently by Dr. A. Lassar's laboratory [74,75]. Other in vitro differentiation systems also show p21 induction [71,74]. This kind of pathways may provide a paradigm for how various cell types undergo terminal differentiation.

Aims 2 and 3: Construction and analysis of mice lacking p21.

Targeted disruption of the *p21* gene creates a null mutation. Pumin Zhang in the laboratory constructed several targeting construct in order to create ES cells lacking p21. Numerous attempts to generate such cell lines resulted only in the identification of ES clones in which there was integration but not double cross-over events. Thus, despite the independent construction of several knockout constructs and numerous attempts to identify EC cells harboring homologous recombinants at the p21 locus, we were unable to identify such clones. As such we established a collaboration with Dr. C. Deng and Dr. P. Leder, who successfully targeted the p21 locus and achieved germline transmission (56). The targeting construct employed, p21neo, deletes exon 2 of the p21 gene, encoding about 90% of the protein. Of 70 G418 and FIAU double resistant embryonic stem cell (ES) clones analyzed by Southern analysis for homologous recombination, one contained a correct targeting event. Germline transmission was obtained from the injection of this ES clone into C57BL/6J blastocysts. Southern blot analysis indicated that about 50% of agouti offspring were heterozygous for the targeted mutation ($p21^{+/-}$).

p21 is expressed in most organs and tissues in a p53-independent manner during murine embryonic and post-natal development (72). To study the possible role of p21 in murine development and in tumorigenesis, mice heterozygous for the targeted disruption were intercrossed to produce homozygous offspring. These crosses gave rise to litters of normal size, with living, apparently normal $p21^{-/-}$ offspring present at a frequency consistent with Mendelian inheritance. $p21^{-/-}$ mice were monitored for evidence of illness or tumor formation weekly up to 7 months and peripheral blood examinations, which showed no evidence of red or white blood cell abnormalities, were carried out at 4 months of age. Histological sections from several organs, such as muscle, testis, vertebral bones, brain, were examined and were found to be normal.

Cultured mouse embryonic fibroblasts (MEFs) were derived from day 14 embryos of the three possible genotypes, wild type, $p21^{+/-}$ and $p21^{-/-}$. These were compared to similarly derived $p53^{+/-}$ and $p53^{-/-}$ embryonic fibroblasts with respect to p21 mRNA and protein (56). Very little p21 mRNA (about 2% of wild type) could be detected in samples from p53-deficient cells. No p21 mRNA was detected in samples from $p21^{-/-}$ cells nor could p21 protein be detected in samples from either p53- or p21-deficient cells Given these results and the extensive nature of the deletion, we conclude that this p21 mutation is a null.

Growth properties of p21-/- cultured embryonic fibroblasts . Proliferation and saturation densities of wild type, $p21^{-/-}$ and $p53^{-/-}$ MEFs were analyzed at two passage points. At lower passages (passage 1 to 3), all of the cells, irrespective of their genotypes, grew quite rapidly and had similar growth rates before becoming confluent (56). All MEFs showed contact inhibition, but the monolayers formed by $p21^{-/-}$ and $p53^{-/-}$ MEFs were more crowded than those formed by wild type cells. In addition, the saturation densities of $p53^{-/-}$ cells were significantly higher than those of wild type cells while those of $p21^{-/-}$ cells were intermediate between those of wild type and $p53^{-/-}$ cells (56).

At later passages (passage 5-15), as growth of wild type cells slowed, differences in the saturation densities between the $p53^{-/-}$ and wild type MEFs became much more pronounced. $p21^{-/-}$ MEFs behaved more like $p53^{-/-}$ MEFs (56). Between passage 5 to 15, proliferation of wild type MEFs was significantly reduced (56). Wild type cells gradually accelerated growth rates between passages 15 to 20, while the behavior of $p21^{-/-}$ and $p53^{-/-}$ MEFs did not change (data not shown).

The role of p21 in p53-mediated G1 arrest. In response to DNA damage, mammalian cells arrest the cell cycle. Although the number of points at which the cycle can arrest have not been fully elucidated, it is clear that there is a block to entry into S phase that is mediated in part by arrest in G1. This cell cycle arrest is dependent upon the p53 gene product. Although it is not clear how p53 achieves cell cycle arrest, it has been suggested that it is through the transcriptional activation of the p21 gene (57,59). To examine the contribution of p21 to the G1 checkpoint, we examined the ability of wild type and mutant embryonic fibroblasts to reduce the population of S phase cells in response to γ irradiation.

Serum starved cells were irradiated and stimulated to enter the cycle by the addition of serum. BrdU was added with serum to allow detection of cells entering S phase. 24 h later cells were harvested and the number having entered S phase were determined by bivariate FACS analysis . Incorporation of BrdU indicates cells in S phase (see the example shown in Fig. 1 A). As indicated in Fig. 1B, wild type cells showed a 50 % reduction in the number of S phase cells relative to unirradiated samples averaged over several experiments. By contrast, p53 defective cells showed nearly complete deficiency in the ability to arrest in G1, with, on average, only 2.8 % reduction in response to γ irradiation. $p21^{-/-}$ cells had a phenotype intermediate between wild type and $p53^{-/-}$ cells, showing, on average, a 20 % reduction in cells entering S phase. These results indicate that p21 partially mediates p53's role in G1 arrest in response to γ irradiation.

In addition to γ irradiation, cells can arrest in G1 in response to other types of damage or metabolic perturbations. N-(phosphonacetyl)-L-aspartate (PALA) is a specific inhibitor of de novo uridine biosynthesis that works through inhibition of the CAD gene . Cells treated with PALA show a p53-dependent decrease of entry into S phase. We examined the role of p21 in cell cycle arrest in response to PALA. Asynchronous cells were treated with varying amounts of PALA for 48 hours and then labeled for 30 minutes with BrdU to identify cells in S phase. Both $p53^{-/-}$ and $p21^{-/-}$ cells display a decrease in the G1 cells population relative to wild type cells and an increase in S phase cells (Fig. 2A).

The large increase in the S phase population is thought to arise from cells that fail to arrest in G1, enter S and become arrested there due to a lack of dTTP caused by PALA. Interestingly, p21 deficient cells show a significant increase in G2 cells that is not observed in p53 defective fibroblasts. This may indicate a p21-independent, p53-dependent function in G2 arrest in response to PALA treatment. At higher concentrations of PALA it becomes difficult to measure precisely the S and G2 contents in $p21^{-/-}$ and $p53^{-/-}$ cells as aberrant patterns of BrdU incorporation are observed in FACS analysis. Nevertheless, it is clear that the $p21^{-/-}$ fibroblasts are defective in the G1 checkpoint activated by nucleotide depletion, thereby implicating p21 as a significant mediator of p53's checkpoint function in response to nucleotide depletion.

Interpretation and quantification of the pulse labeling results on asynchronous cultures are complicated in part by the fact that cells can arrest in G1, G2, or M and thereby prevent S-phase entry. To more precisely measure the effects of PALA on the G1 to S phase transition, we performed experiments using synchronized cells. Serum starved cells were stimulated to enter the cycle in the presence of PALA and BrdU by the addition of serum. 24 h later cells were harvested and the number having entered S phase were quantitated by bivariate FACS analysis (Figure 2B). As observed in asynchronous cells, both $p21^{-/-}$ and $p53^{-/-}$ cells show a nearly complete defect in G1 arrest relative to wild type and $p21^{+/-}$ heterozygous cells. Unlike the results with γ irradiation, the defect in PALA-dependent arrest in $p21^{-/-}$ cells appears to be nearly as severe as the defect with $p53^{-/-}$ cells. This difference may be due to the precise position in the G1 to S phase transition that cells arrest in response to the different treatments, to the additional pathways activated by the stimuli, or to the degree of activation of the p53 protein itself.

<u>p21 is not required for the G2 spindle checkpoint.</u> In addition to the G1 checkpoint, p53 controls a mitotic spindle checkpoint [54]. Wild-type fibroblasts treated with the microtubule inhibitor colcemid arrest with a 4N DNA content. However, cells defective for p53 fail to arrest and proceed through the cell cycle, becoming polyploid. Wild type, $p21^{-/-}$, and $p53^{-/-}$ fibroblasts were examined for their ability to arrest the cell cycle in response to colcemid (Figure 3). While $p53^{-/-}$ cells fail to arrest in the presence of colcemid and accumulate substantial quantities of polyploid cells, $p21^{-/-}$ and wild type cells have intact mitotic checkpoints.

In the absence of spindle inhibitors, 50% of $p53^{-/-}$ MEFs became tetraploid at passage 7 [54] and nearly all $p53^{-/-}$ MEFs became tetraploid or polyploid at passage 9. To determine whether p21 is involved in maintaining the chromosomal ploidy during culture, chromosome numbers were determined for wild type, $p21^{-/-}$ and $p53^{-/-}$ MEFs at passage 3 and 15. The chromosome number

was obtained by counting 50 intact metaphase spreads of each cell type. At passage 3, wild type and $p21^{-/-}$ MEFs had a similar karyotype, i.e. 90% of cells had a typical diploid chromosomal number of 40 and less than 10% cells were tetrapoid. In contrast, 30% of $p53^{-/-}$ cells became tetrapoid or polyploid and an additional 10% contained chromosome numbers between 62 to 78. At passage 15, 30% of wild type and 40% of $p21^{-/-}$ MEFs had 40 chromosomes respectively, whereas >90% of the $p53^{-/-}$ MEFs were aneuploid, tetraploid or polyploid. Thus, p21 is not required for the operation of the mitotic spindle checkpoint.

p21 is not required for p53-dependent apoptosis. Thymocytes readily undergo apoptosis after DNA damage in a p53-dependent manner (52). To determine whether p21 is required for this process, thymocytes were isolated from wild type, $p21^{-/-}$ and $p53^{-/-}$ mice and the percentage of apoptotic cells were determined after γ irradiation. Wild type and $p21^{-/-}$ thymocytes show a dramatic increase of percentages of cells undergoing apoptosis (from about 10% to 60%), whereas the $p53^{-/-}$ thymocytes show only a slight increase of apoptotic cells. Cell death by apoptosis was confirmed by gel electrophoretic analysis of genomic DNA (56). The DNA fragmentation patterns displayed by wild type and $p21^{-/-}$ thymocytes were prominent and indistinguishable, while that of the $p53^{-/-}$ thymocytes was greatly reduced by comparison. Thus it appears that p53-dependent apoptosis in mouse thymocytes does not require p21 .

Absence of accelerated tumorigenesis in p21-/- mice. p53-deficient mice display a marked and rapid rate of early tumorigenesis (44). Since p21 is regulated by p53 and is involved in the G1 DNA damage checkpoint, it is conceivable that $p21^{-/-}$ mice would also display increased rates of tumorigenesis. To date, we have carefully followed 12 $p21^{-/-}$ mice who are now over 7 months of age (and over twenty mice now between the ages of three and four months). In contrast to $p53^{-/-}$ mice over 70% of which develop tumors by six months of age (44), none of the $p21^{-/-}$ mice developed malignancies indicating that p53-dependent suppression of early tumor formation does not require p21.

Obviously, these $p21^{-/-}$ mice will be followed into old age and a propensity for late tumorigenesis may emerge. Moreover, in view of the effect that p21 has on the G1 DNA damage checkpoint, it will be important to assess the radiation sensitivity of these mice. Further, it will be of interest to follow these mice as the $p21^{-}$ mutation is passed into various inbred backgrounds in an effort to discover modulating genes.

Aim 5: Analysis of p21 and associated proteins

p21 associates directly with PCNA and blocks PCNA dependent DNA replication. Previous studies have indicated that p21 in normal cells is present in quaternary complexes containing a Cdk, a cyclin, PCNA, and p21. In contrast, transformed cells have binary Cdk complexes containing a Cdk and a cyclin. Importantly, these transformed cells either lack detectable p21 or have much lower levels of p21. This led us, in collaboration with Dr. J. Hurwitz, to examine whether p21 could associate with PCNA and perhaps bridge PCNA to Cdks.

PCNA is an accessory factor for DNA polymerase delta and is required for DNA replication carried out by pol δ in vitro. We found that addition of pure E. coli derived p21 to PCNA dependent pol δ reactions resulted in a dose dependent decrease in DNA replication (Figure 4).(67) Inhibition was observed both in crude lysates using an SV40-based replication assay and using purified components. Inhibition was independent of addition of cyclin/Cdk subunits and was overcome by addition of excess PCNA (Figure 5). These data indicate that p21 can associate with PCNA and that this interaction can interfere with PCNA's ability to function in DNA replication.

In order to demonstrate directly that PCNA can associate with p21, binding assays were performed using the BIACORE system which detects protein-protein interactions. Pure E. coli p21 was found to associate tightly with PCNA but not with functionally related proteins.(67) Data

from other labs have confirmed and extended these data and have shown that the C-terminal region of p21 are involved in association with PCNA. Similar results were obtained by others [68].

p21 is not detectably phosphorylated in normal diploid fibroblasts. One goal of this work was to determine whether p21 is phosphorylated and if so is it regulated by such modifications. Normal diploid fibroblasts were radiolabeled with either ^{35}S-methionine or ^{32}P inorganic phosphate using standard conditions and then Cdk complexes purified by immunoprecipitation using antibodies against Cdks or p21. Immune complexes were subjected to SDS-PAGE and autoradiography to visualize labeled proteins. While p21 was detected in ^{35}S-methionine labeled extracts, it was not detected in 32P labeled extracts (Figure 6). As a control, ^{32}P was detected in the Cdk proteins. Thus while it has been shown that p21 can be phosphorylated by Cdks in vitro using baculovirus proteins [84], it does not appear to be detectably phosphorylated in intact cells. Importantly, in the in vitro studies, it was shown that phosphorylation of p21 causes a large shift in its mobility to a slower migrating band. These more slowly migrating bands and not evidence in immunoblots of p21 protein in a number of tissue culture cell lines, or in immunoprecipitations from ^{35}S-methionine labeled cells, consistent with a lack of phosphorylation.

p21 is associated with the majority of active Cdk2 in normal fibroblasts. While it is clear that p21 can act as an inducible inhibitor of S phase entry, it is not clear what role it plays in actively growing cells. One possibility is that it functions as a buffer to determine the level of active kinase required for passage through particular cell cycle transitions. To understand the physiological significance of this buffer, it is important to know what fraction of kinase is associated with p21 in vivo. Quantitative immunoblot analysis of WI38 fibroblast extracts was performed using *E. coli* derived HA-p21 and HA-Cdk2 as standards. As shown in Figure 7A, p21 levels are 2-4 fold higher than the total Cdk2 protein, as determined by the relative immunoblot signals of HA-p21 and HA-Cdk2 determined in parallel. This p21 is presumably distributed among multiple Cdks.

To determine what fraction of Cdk2 in normal diploid fibroblasts is bound to p21, we depleted p21 from [^{35}S]methionine-labeled WI38 extracts by sequential immunoprecipitations and then determined what fraction of active Cdk2 remained in the depleted extract using histone H1 as substrate (Figure 7B). As a control for Cdk2 immunoprecipitation and kinase activity, two sequential Cdk2 immune complexes were prepared from an equivalent amount of lysate (Figure 7B, lanes 6 and 7). This experiment revealed that: i) the vast majority of active Cdk2 complexes are associated with p21 in normal diploid fibroblast, and ii) that p21 immune complexes contain substantial levels of H1 kinase activity. The activity of the Cdk2 immune complexes from the undepleted extract was 40% of that contained in p21 immune complexes. In contrast, the kinase activity of the Cdk2 immunoprecipitates from the p21-depleted extract (Figure 7B, lane 5) was essentially indistinguishable from background activity found in normal mouse serum immune complexes (Figure 7B, lane 1).

The observation that active kinases are present in p21 immunoprecipitations is surprising, given the fact that p21 inhibits Cdk activity, and was recently reported by Zhang et al. [84]. This activity could be due to several possibilities. Modified forms of p21 or Cdk/cyclin complexes could exist that can bind each other but do not inhibit kinase activity. Alternatively, unrelated kinases could be present in these complexes as part of a higher order complex containing p21 and Cdks. Both of these explanations predict that the kinase activity in p21 immune complexes would be resistant to inhibition by exogenous p21. However, since exogenous p21 can block the p21-associated H1 kinase activity (Figure 7B, lanes 8 and 9), both possibilities are eliminated. We also considered the possibility that kinase activity was due to re-equilibration of immune complexes after isolation as a result of changing the buffer composition. As shown in Figure 7C, greater than 93% of the anti-p21 associated kinase activity remained associated with anti-p21 immune complexes under the conditions of the kinase assay, ruling out re-equilibration as an explanation for kinase activity in p21 containing complexes.

An alternative explanation is that p21 can associate with Cdks in a non-inhibitory mode. For example, inhibition could require association of multiple p21 molecules, with association of

the first molecule being non-inhibitory, as suggested recently by experiments utilizing crude insect cell lysates [84]. In this scenario, p21 immune complexes from cell extracts would contain a mixture of inhibited and active Cdks, depending upon the relative levels of p21 and cyclin associated Cdks in the particular extract. In order to test this hypothesis, the kinase activity of anti-HA immune complexes generated from samples containing a constant amount of Cdk2/cyclin A and varying amounts of HA-p21 was examined (Figure 8A,B). For these experiments, HA-p21 purified from *E. coli* and [^{35}S]methionine-labeled Cdk2/cyclin A, purified to apparent homogeneity from sf9 cells, was used. This Cdk2/cyclin A preparation migrated at the expected position for a monomeric complex using gel filtration (Figure 8C). The use of pure proteins avoids potential artifacts which could come into play when crude sf9 extracts are used.

As shown in Figure 8A, increasing amounts of Cdk2/cyclin A were immunoprecipitated with increasing amounts of HA-p21. Quantitation of labeled proteins revealed that the maximum amount of Cdk2/cyclin A recovered with anti-HA antibodies was ~60% of that recovered with anti-Cdk2 antibodies. At the lower concentrations of HA-p21 used, substantial histone H1 kinase activity was observed in these immune complexes (Figure 8B), but as the concentration of HA-p21 was increased, the Cdk2 kinase activity diminished sharply. The specific activity of the HA-p21/Cdk2/cyclin A complex associated with the lowest amounts of HA-p21 was at least 20% of that of the Cdk2/cyclin A complex, based on the activity of the anti-Cdk2 immune complexes assayed and quantitated in parallel (Figure 8A). Interestingly, at 70 nM HA-p21 approximately 30 nM Cdk2 was precipitated and this complex is essentially inactive. These results rule out bridging molecules as being involved in p21 associated kinase activity and are consistent with the idea that multiple molecules of p21 can associate with Cdk/cyclin complexes [84].

Conclusion:

Since the discovery of p21 as a p53-inducible gene, a number of functions have been attributed to it. These include cell cycle arrest in response to DNA damage, apoptosis, cell cycle arrest during differentiation, and as a possible tumor suppressor. We have shown that p21 is selectively expressed during embryonic development and in adult organs and the pattern of expression correlates with terminal differentiation (Aim 1). p21 expression in vivo is not dependent upon p53, indicating that other mechanisms can function to activate p21 expression. In order to understand the role of p21 more fully, we have examined the role of p21 in a number of different processes through the analysis of mice lacking the p21 gene (Aims 2 and 3). The results demonstrate that p21 is required for only a subset of p53's functions and is not generally required for cellular differentiation (see Figure 9), although we cannot exclude a subtle role for p21 that is not readily observed phenotypically. p21 is not required for thymocytic apoptosis or for the p53-dependent G2 checkpoint but is required for the G1 checkpoint in response to both ionizing radiation and nucleotide pool perturbation. Mice lacking p21 have been followed for 9 months with no evidence for early tumors of any type. This is in stark contrast with p53-deficient mice where greater than 70% of mice incur tumors by four months of age. Thus, the data accumulated to date suggest that p21 is not a tumor suppressor and, alone, is not responsible for p53's tumor suppressor function. It is possible that p53's apoptotic function or a combination of checkpoint and apoptotic functions are critical to p53's tumor suppressor function.

In addition to these biological studies, we have performed a number of experiments to determine the specificity of p21 toward various Cdk family members, have shown that p21 can exist in both active and inactive Cdk complexes, and have identified PCNA as a p21 associated protein (Aim 5). Association of p21 with PCNA blocks its ability to function as a cofactor for pol delta dependent DNA replication in vitro. It is not clear however whether this function is critical to its cell cycle arrest function. One aim of our work (Aim 4) was to determine whether p21 was mutated in breast and other cancers. Previously, we found that p21 was expressed in normal breast epithelial cell strains but was absent in a number of tumor derived cell lines. A number of laboratories have looked for mutations in the p21 gene but no mutations have been

identified. This is consistent with the results of the p21-deficient mice which indicate that p21 is not a tumor suppresser in the classical sense.

Future goals and new directions. An important issue relates to the question of whether loss of p21 expression may in some way potentiate tumor formation or alter the spectrum of tumor types. While p21 loss by itself may not lead to direct transformation, it is likely that within the context of another mutation it will function as a tumor suppressor. Potential classes of mutations that may lead to altered transformation frequency of cell type specificity include overexpression of cyclins such that the balance of negative and positive regulators is disturbed, or overexpression of myc. In the coming years, we will attempt to generate doubly mutant mice in which candidate mutant genes are combined with p21 mutations. One of the major targets will be cyclin D1 which has been shown to cause transformation of mammary cells in vivo when placed under control of the MMTV promoter. We will specifically ask whether the frequency of mutation is altered or whether there are changes in tumor type. Eventually, it may also be possible to mate p21 deficient mice with mice lacking other CKIs such as p27 or p16 which are currently being constructed in other laboratories. In addition, we will further analyze phenotypes associated with loss of p21 as there may be subtle phenotypes which emerge upon close examination of the animals.

A second area relates to the alternate G1 checkpoint pathway that we have uncovered without analysis of the p21 deficient fibroblasts. Available data suggests that tyrosine phosphorylation of Cdk4 may contribute to cell cycle arrest in G1 in response to UV irradiation. We will attempt to examine alternative mechanisms in greater detail both biochemically and in vivo. We will attempt to created transgenic mice which express a mutant Cdk4 protein that cannot be tyrosine phosphorylated in combination with loss of p21. The prediction would be that this animal would have a more severe G1 checkpoint defect and would therefore potentially be predisposed to tumor formation.

An additional area of research which may be fruitful is an analysis of the expression of p21 during mammary cell differentiation in vivo. This can be accomplished using in situ hybridization from virgin, pregnant, and lactating mice.

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Appendix

Tables 1-3.

Figures 1-9.

Figure Legends

Figure 1. Mouse Embryonic Fibroblasts Lacking $p21^{CIP1/WAF1}$ Display a Defect in the Ability to Block S-phase Entry After γ-Irradiation

(A) A representative bivariate flow cytometric analysis of synchronized wild type (WT), $p21^{-/-}$, or $p53^{-/-}$ MEFs 24 h after exposure to 0 or 20 GY γ-irradiation. Serum starved cells (4 days in media containing 0.1% FBS) were released into complete media containing BrdU (65 μM) and immediately irradiated. At 24 h after release, cells were harvested, and stained for DNA content with propidium iodide and for replicative DNA synthesis with a fluorescein isothiocyanate-conjugated anti-BrdU antibody. Boxes labeled 1, 2, and 3 indicate G1, S and G2/M phase cells, respectively, and the percentages of cells in each phase of the cycle indicated for each diagram. Approximately three thousand cells were examined in each analysis.
(B) Quantitative analysis of the effects of γ-irradiation on the number of cells entering S-phase for WT, $p21^{-/-}$, and $p53^{-/-}$ MEFs. The percentage of cells entering S-phase (BrdU-positive) after irradiation relative to unirradiated cells are shown along with standard deviations for replicate experiments. n refers to the number of independent determinations for each strain.

Figure 2. Absence of a Functional PALA-induced G1 Checkpoint in $p21^{-/-}$ MEFs

(A) Effects of PALA on the cell cycle distribution of asynchronous MEFs. Asynchronous MEFs (passage 3) of the indicated genotypes were treated with various concentrations of PALA for 48 h. Cells were pulse-labeled with BrdU (30 minutes), harvested, and analyzed for DNA synthesis and DNA content by FACS analysis as described for Figure 3. Below is the quantitation of cell cycle phase distribution for these cells after PALA treatment.
(B) Effects of PALA on S-phase entry of synchronized MEFs. The indicated MEF cultures at passage 3-4 were serum starved for 84 h prior to addition of the indicated concentrations of PALA for 12 h and then the cells were released into growth media containing the same concentrations of PALA and BrdU (65 μM). At 24 h, cells were harvested and subjected to bivariate flow cytometric analysis. The percentage of cells entering S-phase (BrdU-positive) in the presence of PALA normalized to the number of cells entering S-phase in the absence of PALA are shown. The percentage of cells entering S-phase in the absence of PALA were 41, 32, 60, and 82 for WT, $p21^{+/-}$, $p21^{-/-}$, and $p53^{-/-}$, respectively.

Figure 3. The p53-dependent spindle checkpoint induced by colcemid is functional in $p21^{-/-}$ MEFs.

Asynchronous wild-type, $p21^{-/-}$, and $p53^{-/-}$ MEFs at passage 3 were incubated in the absence or presence of 500 ng/ml colcemid for 24 h. Cells were harvested and the DNA content determined by flow cytometry after DNA staining with propidium iodide. DNA content flow cytometric histograms are shown as are the positions of peaks for 2N, 4N, and 8N DNA content. A minimum of 3000 cells were examined in each experiment.

Figure 4. p21 inhibits PCNA dependent SV40 DNA replication in vitro.

Replication of SV40 DNA by crude extracts was carried out in reaction mixtures containing 300 ng of SV40 plasmid DNA, 830 ng of T-antigen, 300 ng of HSSB, 0.18 mg of Hela nuclear extract, and p21 as indicated. Reactions were incubated for 90 min and the amount of acid-insoluble 32P measured.

Figure 5. Excess PCNA can overcome p21's ability to block DNA replication through PCNA association.

Reaction mixtures containing 10 mM Tris, 10 mM creatine phosphate, 1000 ng creatine phosphokinase, 2 mM DTT, 0.03 mM 32P-dTTP, 2 mM ATP, 300 pmol polyA-oligodT, 600 ng HSSB, 0.2 units of RFC, 0.1 unit of pol-delta, and PCNA and p21 as indicated. Acid insoluble poly dT was measured.

Figure 6. p21 is not detectably phosphorylated in WI38 fibroblasts.

Growing fibroblasts were radiolabeled with either 35S-methionine or 32P inorganic phosphate and extracts subjected to immunoprecipitation using anti-21 antibodies of p13 beads. Immune complexes were subjected to SDS-PAGE and autoradiography. p21 excessed in insect cells was used as a control.

Figure 7. p21 is associated with the majority of active Cdk2 in WI38 fibroblast extracts.

(A) Quantitative immunoblot analysis of p21 and Cdk2 in extracts from WI38 cells. A dilution series of the indicated quantities of HA-p21 and HA-Cdk2 were subjected to immunoblotting along with a 10 µl aliquot of WI38 cell extract (equivalent to ~210,000 cells) and the blot probed with anti-Cdk2 and anti-p21 antibodies. Detection was accomplished using ECL. Assuming similar transfer efficiency for p21 and Cdk2, the molar ratio of p21 to Cdk2 is 2-4. (B) Extracts from [^{35}S]methionine labeled WI38 cells were immunoprecipitated sequentially with either normal mouse sera (NMS), anti-p21, or with anti-Cdk2, as indicated. Extracts corresponding to one 10-cm dish were used for each immunoprecipitation. Twenty percent of each immune complex was used for histone H1 kinase assays (30 min., 37°C) and the remaining immune complex separated by SDS-PAGE prior to autoradiography. A third aliquot of unlabeled fibroblasts (extract corresponding to 1/2 of a 10-cm dish) was subjected to immunoprecipitation with anti-p21 and the immune complexes divided equally. To one of the two immune complexes, 500 nM p21 purified from E. coli was added. Histone H1 kinase assays were performed as described above. Histone H1 kinase activities were assessed by SDS-PAGE and autoradiography. (C) The majority of active Cdk2 remains associated with anti-p21 immune complexes during the kinase assay. WI38 lysates (500 µg protein) were immunoprecipitated with anti-p21 mIgG (in duplicate) or with NMS and immune complexes prepared for kinase assays. One anti-p21 complex and the negative control complex were kept on ice (30 min) while the second anti-p21 complex was incubated at 37°C in the presence of 1 µM histone H1 but without ATP. After 30 min, the supernatant from the mock kinase reaction was removed to a new tube, the immune complex reconstituted with kinase buffer, and H1 kinase reactions performed on all of the immune complexes in the presence of ^{32}P-ATP (30 min, 37°C). Reactions were analyzed by SDS-PAGE and and quantitated by phosphoimager analysis.

Figure 8. Association of active forms of Cdk2/cyclin A with p21.

(A) A constant amount of purified [^{35}S]methionine-labeled cyclin A/Cdk2 (~50 ng Cdk2) was incubated with increasing amounts of HA-p21 purified from *E. coli* (see panel B for concentrations) in a total volume of 30 µl of EB. After 10 min., 3 µg of anti-HA antibodies, 10 µl protein A-Sepharose, and 150 µl of binding buffer were added prior to immunoprecipitation. As a control, anti-Cdk2 immunoprecipitations were carried out on ~50 and ~5 ng of cyclin A/Cdk2. Eighty percent of the immune complex was subjected to SDS-PAGE and autoradiography (A) while the remainder was assayed for kinase activity using histone H1 peptide as substrate (B). The activity of the immune complex derived from 5 ng Cdk2 gave 350,000 cpm. Based on the quantitation of ^{35}S-labeled Cdk2 present in the anti-HA and anti-Cdk2 immunoprecipitates using phosphoimager analysis, the maximum specific activity for the anti-HA complex was 20% of that of Cdk2/cyclin A (at 7 nM HA-p21). (C) Cyclin A/Cdk2 migrates as a monomeric complex on a gel filtration column. Approximately 3 µg of purified cyclin A/Cdk2 (in 0.2 ml column buffer)

was chromatographed on a Superose-12 column. Two µl aliquots were assayed for histone H1 peptide activity (1 h, 37°C). The elution positions of molecular weight markers are shown.

Figure 9. A Current View of the Integrated Actions of p21 and p53

DNA damage leads to stabilization and activation of p53, possibly via the ATM gene product. The activated p53 protein (p53*) induces transcription of $p21^{CIP1/WAF1}$ and other genes involved in cell cycle arrest and DNA repair. Under certain conditions that are not completely understood, p53 activation can lead to apoptosis. In response to γ irradiation, p53-dependent cell cycle arrest in G1 functions by both $p21^{CIP1/WAF1}$-dependent and independent mechanisms that are only partially redundant, while p53-dependent apoptosis in response to γ irradiation does not require $p21^{CIP1/WAF1}$. In mouse cells, p53 also regulates re-entry into S-phase when mitosis is blocked, as in the case of the anti-microtubule agents colcemid and nocodazole (54). This function is independent of $p21^{CIP1/WAF1}$. p53 may also regulate ploidy in the absence of spindle interference because untreated $p53^{-/-}$ cells rapidly increase ploidy with increased passage.

Table 1. Mammalian Cdk Inhibitors

Inhibitor	Primary Target	Chromosomal Location	regulator(s)	Comments
Ankyrin Family				
$p15^{INK4b}$	Cdk4,6	9p21	TGFβ	frequently deleted in glioblastoma
$p16^{INK4a}$	Cdk4,6	9p21	?	tumor suppressor (melanoma)
$p18^{INK4c}$	Cdk4,6	1p32	?	not yet associated with cancer
$p19^{INK4d}$	Cdk4,6	19p13	?	not yet associated with cancer
Dual-Specificity Family				
$p21^{CIP1/WAF1}$	Cdk2,3,4,6	6p21	p53,TGFβ MyoD	functions in G1 checkpoint
$p27^{KIP1}$	Cdk2,4,6	12p12-13	cAMP Rapamycin	not yet associated with cancer
$p57^{KIP2}$	Cdk2,3,4	11p15.5	?	candidate tumor suppressor for both sporadic and familial cancers

Table II. p21 expression during embryonic development

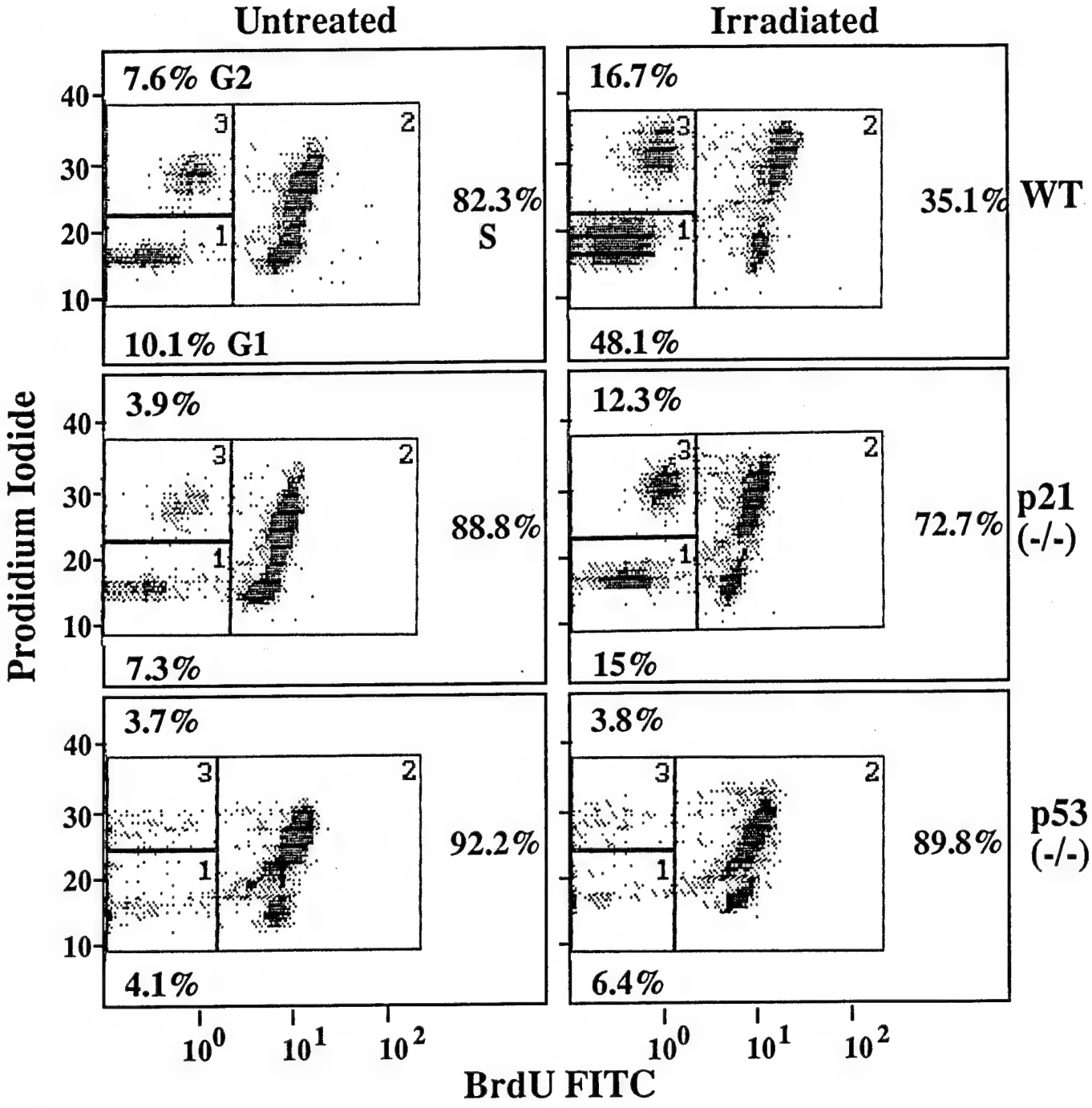
d.p.c.	tissue
7.5	no expression detected
8.5	somites
9.5	dermomyotome apical ectodermal ridge limb bud musculature
12.5	outer layers of the epidermis cartilage skeletal muscle including tongue
15	outer layer of the epidermis respiratory epithelium sensory epithelium hair follicles skeletal muscle including tongue

Table III. Expression of p21 in adult organs

tissue
cardiac muscle
olfaction bulb
columnar epithelium of the stomach and intestine
lung
liver
skeletal muscle
kidney
testis

Figure 1

A

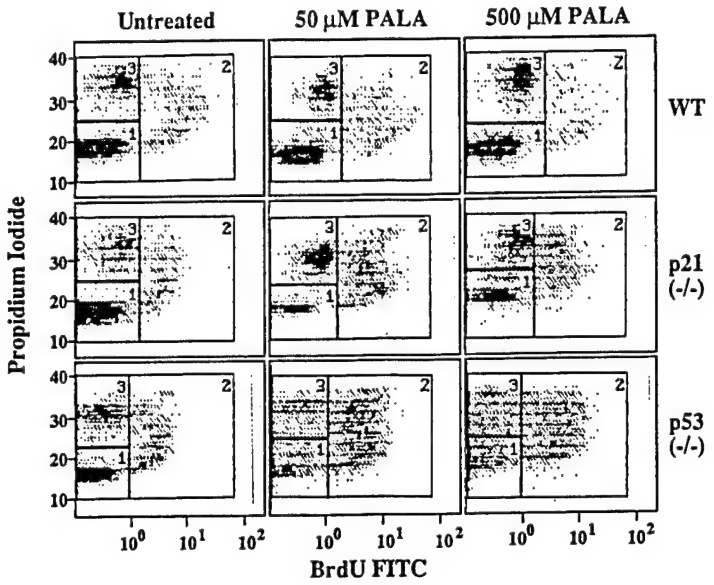


B

Relative % of S-phase Cells $\left(\frac{\text{irradiated}}{\text{unirradiated}} \times 100\right)$		
WT	50.0 ± 3.8	(n = 4)
p21(-/-)	79.8 ± 2.0	(n = 4)
p53(-/-)	97.2 ± 4.6	(n = 3)

Figure 2

A



Strain	[PALA]	0	50µM	500µM
WT	G1	60.1	64.3	58.3
	S	14.5	13.4	6.1
	G2	25.4	22.3	35.6
p21(-/-)	G1	62.7	19.5	29.8
	S	14.7	40.0	24.6
	G2	23.4	40.4	45.5
p53(-/-)	G1	57.8	19.4	23.1
	S	18.6	56.8	44.9
	G2	23.6	23.8	32.0

B

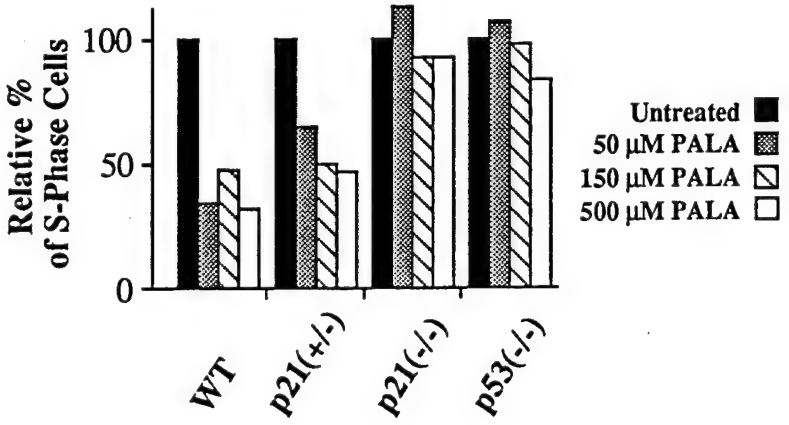
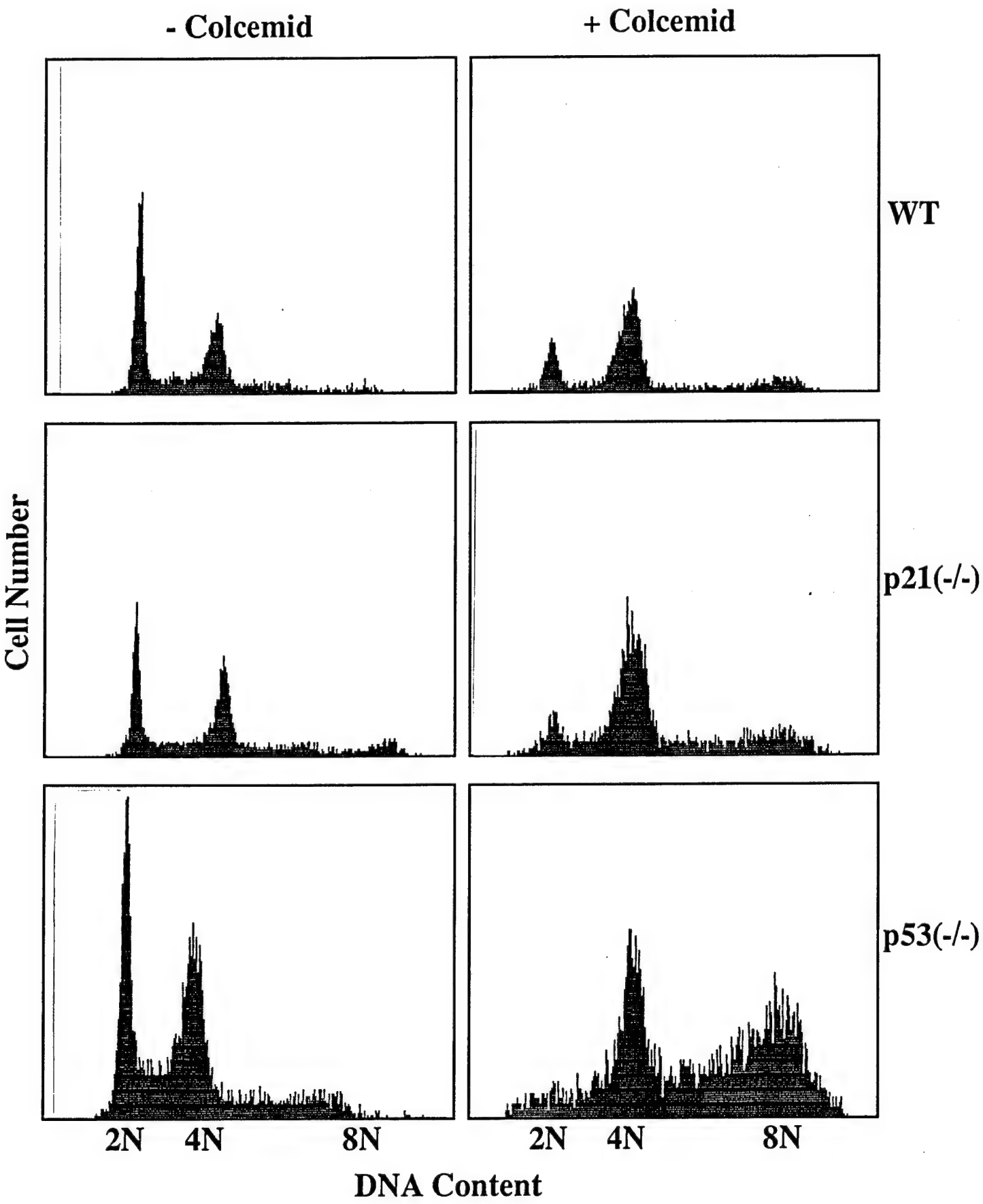
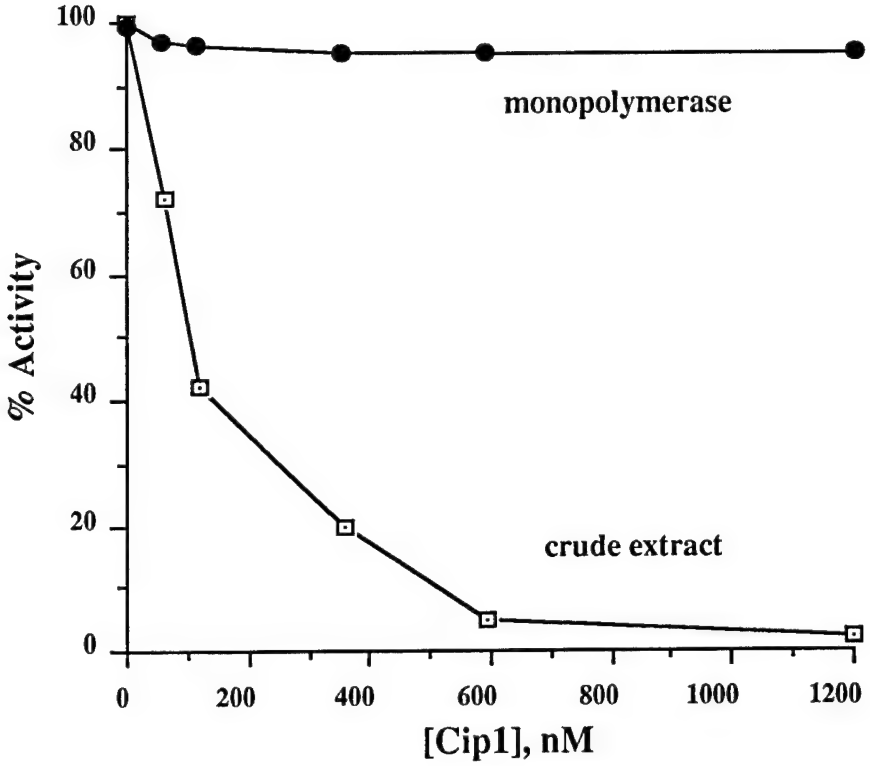


Figure 3

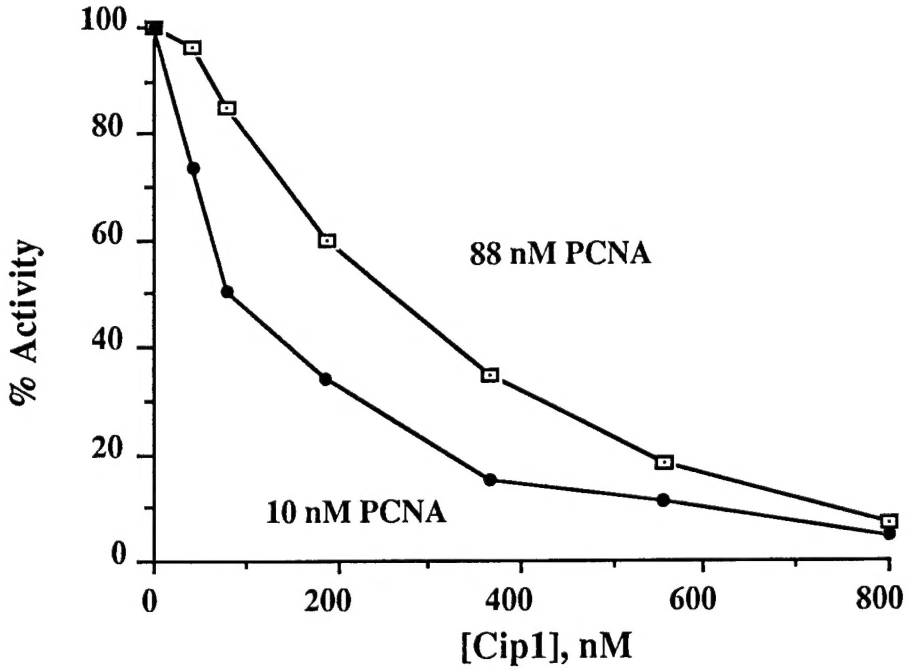

- Colcemid
+ Colcemid
WT
p21(-/-)
p53(-/-)
Cell Number
2N
4N
8N
2N
4N
8N
DNA Content

Cip1 Inhibits SV40 Replication in Crude Extracts but not Replication by the Purified Pol α-primase Complex



Replication of SV40 DNA by crude extracts was carried out in reaction mixtures (40 µl) containing 300 ng of pSV01ΔEP, 830 ng T antigen, 300 ng HSSB, 186 µg crude Hela extract , 32P-dCTP and Cip1 as indicated. Reactions were incubated for 90 min at 37 C and the amount of acid-insoluble counts measured. For the monopolymerase system, reaction mixtures contained SV40 DNA, T-antigen, and HSSB as above plus purified pol α-primase (0.4 and 1.1 units respectively), 12 ng topoisomerase, and 3 µg serum albumin.

Cip1 Inhibits pol δ/RF-C Dependent Replication in a PCNA Concentration Dependent Manner



Reaction mixtures (30 µl) containing 10 mM Tris (pH 7.8), 10 mM creatine phosphate, 1 µg creatine phosphokinase, 2 mM DTT, 150 µg/ml BSA, 30 µM 32P-dTTP, 2 mM ATP, 7 mM magnesium chloride, 300 pmol polydA-oligodT, 600 ng HSSB, 0.2 unit A1 (RF-C), 0.1 unit pol δ, PCNA and Cip1 as indicated (20 min, 37 C). Acid insoluble poly dT formed was measured.

Cip1 Is Not Detectably Phosphorylated in Fibroblasts

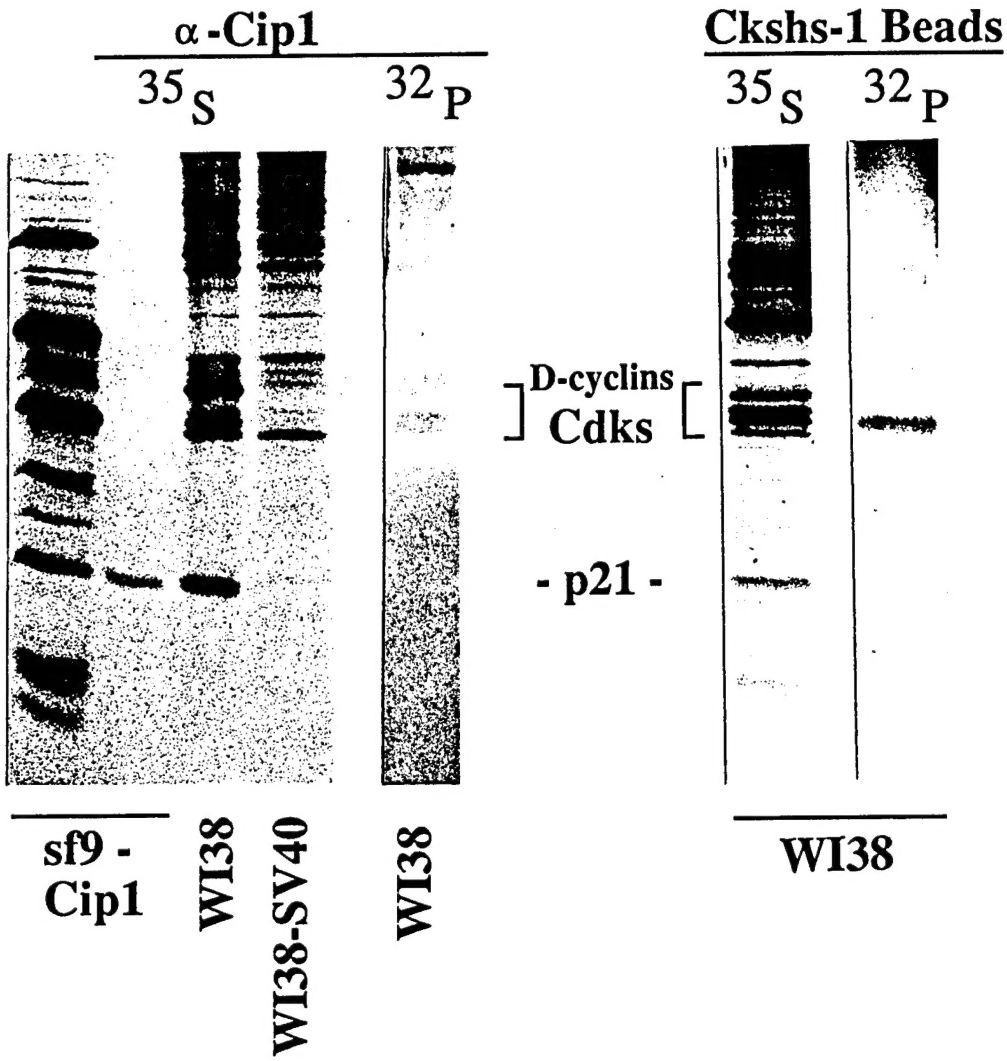


Figure 7

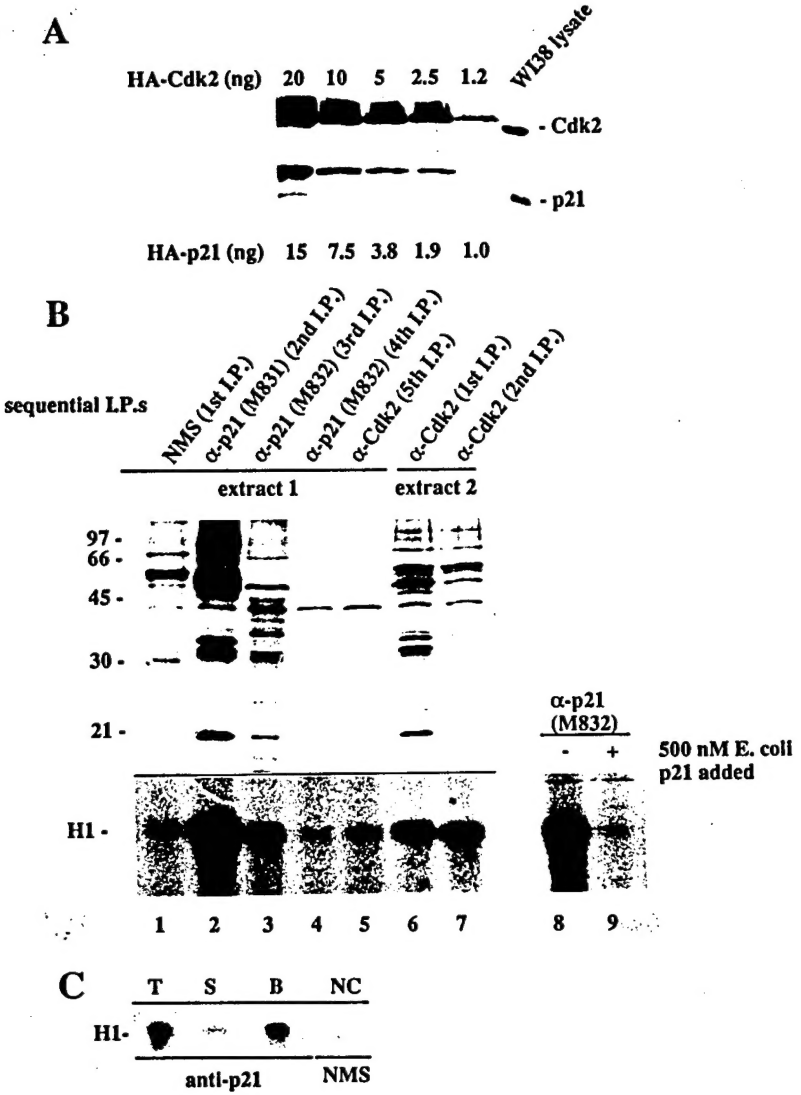


Figure 8

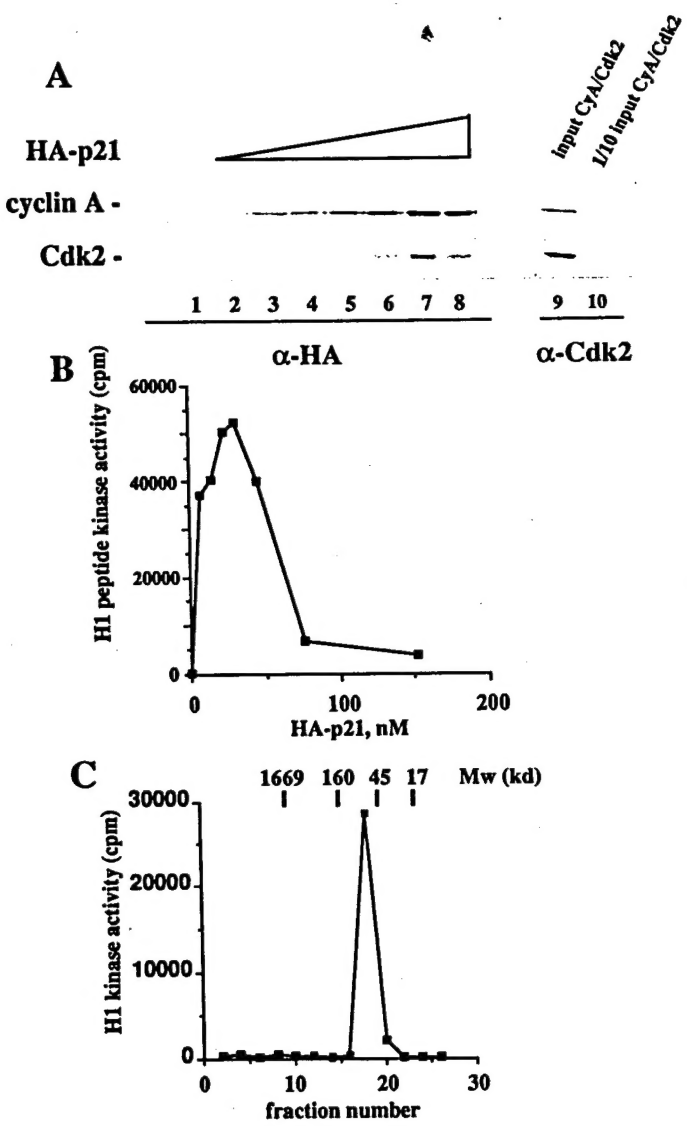


Figure 9. A Current View of the Integrated Actions of p21 and p53